





Article

Tuber mesentericum and *Tuber aestivum* Truffles: New Insights Based on Morphological and Phylogenetic Analyses

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Abstract: *Tuber aestivum*, one of the most sought out and marketed truffle species in the world, is morphologically similar to *Tuber mesentericum*, which is only locally appreciated in south Italy and north-east France. Because *T. aestivum* and *T. mesentericum* have very similar ascocarp features, and collection may occur in similar environments and periods, these two species are frequently mistaken for one another. In this study, 43 *T. aestivum* and *T. mesentericum* ascocarps were collected in Italy for morphological and molecular characterization. The morphological and aromatic characteristics of the fresh ascocarps were compared with their spore morphology. Afterwards, we amplified and sequenced the elongation factor 1- α (EF1 α) locus and built maximum likelihood trees to assess phylogenetic similarities between the two species. *Tuber aestivum* and *T. mesentericum* sequences cluster into different clades, with *T. mesentericum* sequences divided into three different sub-clades. According to their morphological features, three samples (T7, T8 and T12) were classified as *T. mesentericum*. However, when fresh, these ascocarps lacked the typical phenolic aromatic note. These specimens fall into the sub-clade III of the *T. mesentericum* phylogeny, which has the lowest genetic distance from the *T. aestivum* clade.

Keywords: *Tuber mesentericum*; phylogeny; truffle; ascocarp; *Tuber aestivum*

1. Introduction

Among species belonging to genus *Tuber*, the summer truffle (*Tuber aestivum* Vittad.), is one of the most marketed and cultivated in Europe. This truffle is less valued than the Périgord black truffle (*Tuber melanosporum* Vittad.) and the white truffle (*Tuber magnatum* Pico), but its soil and climatic requirements make it capable of colonizing many different areas, even outside its geographical center of distribution [1]. These features make the summer truffle the easiest species to cultivate. Interest in this species also derives from the long ripening period that goes from May to September (*T. aestivum*) and from September to nearly all through winter (*T. aestivum* var. *uncinatum* (Chatin) I.R. Hall, P.K. Buchanan, Y. Wang & Cole) [2–4], prolonging the supply to the market. Nowadays, *T. aestivum*

mycorrhized seedlings are routinely produced by specialized nurseries, favoring its cultivation to spread all over Europe and more recently in Australia and New Zealand [1,5–12].

Tuber aestivum is morphologically similar to *Tuber mesentericum* Vittad., also known as the “black truffle of Bagnoli Irpino”, a species only locally appreciated in southern Italy and in north eastern France. This truffle is well-distributed in Europe; it can be collected in summer, but the peak of production is between the end of autumn and the beginning of winter. Because of the low market request, the cultivation of this truffle has never taken place. Nevertheless, proof of successful mycorrhization has been provided, opening new possibilities for *T. mesentericum* cultivation [13]. In the wholesale market, and for industrial use, *T. aestivum* is sold in large quantities at prices generally lower than 100 €/kg. While at the retail level, big ascocarps of *T. aestivum* var. *uncinatum* can reach up to 600 €/kg [11]. *Tuber mesentericum* commerce consists of lower quantities than *T. aestivum*. Locally, it can be sold at a price ranging from 100 to 200 €/kg [14]. Differences in prices between the two truffle species are mainly determined by their different aroma. Indeed, the strong and pungent aroma of *T. mesentericum*, frequently associated with an unpleasant note of phenol, tar, and/or iodine, is not commonly appreciated [14].

Tuber aestivum and *T. mesentericum* ascocarps are often mistaken for one another because of their very similar morphology and because the aroma of *T. mesentericum* can, sometimes, lack the phenolic smell that is a unique trait of this species. Moreover, *T. aestivum* var. *uncinatum* and *T. mesentericum* can occur in the same environment and ripen at the same time of the year [15], so a mix of these species can be collected indistinguishably. The many overlapping morphological features of the ascocarps make it possible to refer to *T. mesentericum* and *T. aestivum* as a “species-complex” [16]. The transverse streaks in the peridium wart faces can not be considered a distinguishing feature between the two species because they are shown to be present in both *T. mesentericum* and *T. aestivum*, even if they are less evident and frequent in *T. mesentericum* [14]. Similarly, the presence of a basal depression or cavity is not a valid taxonomic trait to distinguish *T. mesentericum* because ascocarps of *T. aestivum* with the basal cavity, and *T. mesentericum* without it, have been described in the literature [14]. The color of the gleba can be considered a more reliable morphological feature to distinguish the two species. The *Tuber mesentericum* gleba is typically dark grey or brown, often with violet shades and with numerous white intensively winding veins at full maturity. In contrast, the color of the *T. aestivum* gleba ranges from yellowish or light brown to ochre, but never dark brown with violet shades [17,18]. The high intra and inter-specific variability between these two truffles also involves spore size and morphology. In both species the epispore is reticulate–alveolate with polygonal meshes often containing a crest on the inside. In *T. aestivum*, the spore meshes are larger, and basically regular and complete, whereas they are generally incomplete and irregular in *T. mesentericum*. However, also in the latter species (and sometimes even in the same ascocarp), spores with complete meshes, similar to those of *T. aestivum*, can be observed [14]. In both species, spore shape can vary from ellipsoid to perfectly globose and this variability can be found in the same ascocarp. Ellipsoid ascospores are predominant, with a small but variable percentage of spherical and sub-spherical ascospores that are more frequent in “typical” *T. mesentericum* ascocarps [17,18]. The spherical and sub-spherical ascospores can be predominant in some specimens that, based on this feature, have been attributed to distinct species such as *Tuber bellonae* Quél. (synonym: *Tuber bituminatum* var. *sphaerosporum* Ferry de la Bellone) and *Tuber sinoaestivum* J.P. Zhang & P.G. Liu [19,20]. The existence of a unique group for the *T. aestivum*–*T. mesentericum* complex, which also includes *T. bellonae*, is suggested by recent studies based on molecular data [20]. Whereas specific PCR primers to selectively amplify the *T. aestivum* internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (nrDNA) have been developed [21], a similar molecular tool to characterize the whole genotypic diversity of *T. mesentericum* is still lacking due to the high similarity of the two species at that locus [22,23].

Collections of mature *T. mesentericum* specimens (on the order of some tens of kilos), absolutely free of a phenolic aromatic component, are frequently observed from Salento (Puglia, southern Italy) and the province of Rome in late spring and early summer (E.S., personal communication). These sites

of collection are characterized by a Mediterranean climate and are dominated by *Quercus ilex* L. and other *Quercus* host species. These harvests are normally identified by truffle hunters as *T. aestivum*, mostly because they ripen early in the season compared to *T. mesentericum* and because of the absence of the phenolic smell. However, the dark color of the gleba and the morphology of the spores suggest that these specimens are *T. mesentericum*.

In this work we aimed to: (1) expand the molecular and ecological characterization of *Tuber aestivum* and *Tuber mesentericum* and investigate their genotypic diversity using the elongation factor 1- α (EF1 α) locus; (2) compare the molecular data with the detailed morphological characterization of the truffle specimens to confirm the existence of a “*Tuber aestivum*–*mesentericum* complex” and with ecological data to investigate genotype-site relationships; and (3) study *T. mesentericum* ascocarps, which have the aroma most similar to *T. aestivum*, and evaluate their potential in cultivation and trade.

2. Materials and Methods

At the end of 2014, a total of 43 truffles belonging to *T. aestivum* and *T. mesentericum* were collected from 10 different Italian regions (Figure 1). For each truffle, the following information was stored in a database: truffle hunter, place and date of collection, the species name attributed by the hunter, and remarkable features. In particular, we registered if the truffle hunters detected the presence of the phenolic component in the fresh truffles.

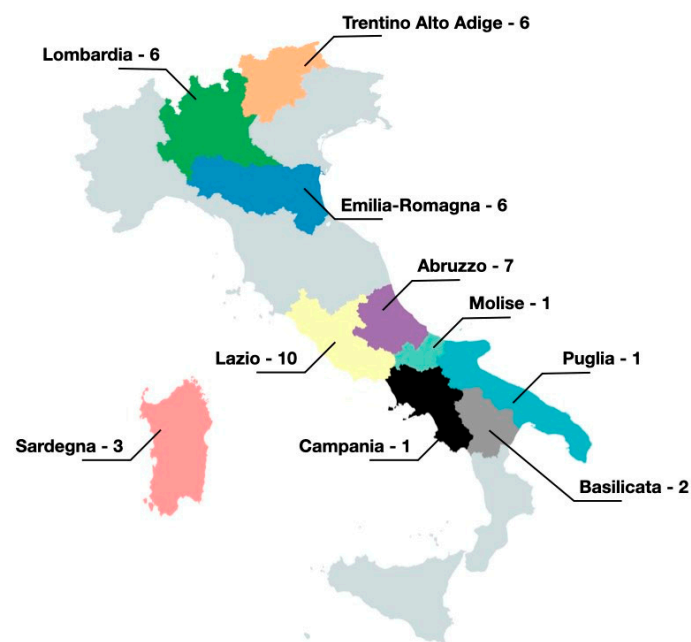


Figure 1. Geographical sampling distribution.

From each truffle, eight gleba cubes of around 0.25 g each were sampled and stored in a 1.5 mL Eppendorf tube containing 70% ethanol (*v/v*) to preserve the DNA for further analysis. At the same time, a portion of fresh gleba was scratched with a scalpel from each truffle and mounted on a microscopic slide with glycerol to perform spore morphological analysis. Details about the truffle samples analyzed in this study are reported in Table 1. The morphological characterization of *T. aestivum* and *T. mesentericum* spores was performed with a light microscope (Leica Leitz DMRB), photographs were taken with a Leica DFC320 digital camera (Figure 2) [24]. Twenty-seven truffles were chosen as representative of the collection sites and used for genetic analysis in order to compare the molecular information with the other available data (morphological analysis of the spores and characterization of the perceived aroma). For each sampled truffle, total DNA was extracted from about 100 mg of grinded material by using the MagCore® Automated Nucleic Acid Extractor and following the protocol for the

MagCore Genomic DNA Plant kit (RBC Bioscience, Taipei, Taiwan). DNA samples were quantified, and quality checked using the NanoDrop (Thermo Scientific, Waltham, MA, USA). The elongation factor (EF1 α) was PCR amplified with a set of primers with enhanced specificity for *Tuberaceae* [25]. Each 45 μ L PCR reaction consisted of 1 \times DreamTaq Buffer, 10 μ g bovine serum albumin (BSA), 0.2 mM dNTPs, 10 μ M of EF1 α Tuber_f (5' AGCGTGAGCGTGGTATCAC 3'—forward) and EF1 α Tuber_r (5' GAGACGTTCTTGACGTTGAAG 3'—reverse) primers, 2 U DreamTaq DNA Polymerase (Thermo Scientific, Waltham, MA, USA) and ultrapure water up to the final volume. DNA in all samples was higher than 10 ng/ μ L and 5 μ L was used to get a satisfactory amplification.

The PCR thermal profile included 5 min at 95 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 60 °C and 45 s at 72 °C and a final extension of 7 min at 72 °C. Three microliters of each PCR product were added to 1 \times DNA Gel Loading Dye and analyzed on a 1.8% (*w/v*) agarose gel stained with ethidium bromide. The PCR products were then purified using the EuroGOLD Cycle-Pure kit (EUROCLONE, Pero, Italy), Sanger sequenced using the BigDye terminator V3.1 kit (Thermo Scientific, Waltham, MA, USA), and analyzed on a ABI PRISM 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Assembled sequence contigs (Geneious v10.2.6) were used as queries against the GenBank [26] database using Basic Local Alignment Search Tool (BLAST) algorithm [27] available on the NCBI [28] portal (Table 1). The resulting contigs were also published in NCBI with accession numbers from MH992040 to MH992066.

All the available EF1 α sequences of *T. mesentericum* and *T. aestivum* were also downloaded from GenBank (Table S1). Sequences were aligned using the ClustalW algorithm then the phylogenetic tree reconstruction was performed using the maximum likelihood method based on the Kimura 2-parameter model [29]. Two phylogenetic trees were constructed: the first one, included sequences produced in this work; the second one, included also sequences downloaded from GenBank. Both trees were supported by bootstrap (999 replicates) and EF1 α sequences from *Tuber magnatum* Pico [25] were included as an outgroup in the first tree. The model used for the phylogenetic reconstruction was computed using the statistical J Model Test. All these analyses were conducted in MEGA7 [30]. The second tree was visualized with iTOL [31].

For sequences produced in this work, the number of base substitutions per site, from averaging over all sequence pairs, between groups identified in the phylogenetic analysis were calculated together with relative standard errors. The within groups average number of base substitutions per site were also calculated.

To assess the level of correspondence between the phylogenetic and the ecological traits (Table S2) of the samples, we performed a tanglegram analysis using the “dendextend” package in R [32,33]. This represents a simple method to visually compare distance matrices having the same set of samples [34]. The quality of the alignment of the two trees is measured via the entanglement that varies between 1 (full entanglement) and 0 (no entanglement); a lower entanglement coefficient corresponds to a good alignment. Penalty was assessed using $L = 1$, whereby increased L results in an increased penalty for sharp angles. A Mantel test was performed in “vegan” R package [35] to assess the correlation between phylogenetic and ecological distance matrices.

Table 1. List of collected samples, including: sample ID, recognized species by ascocarp morphology (*Tuber aestivum* var. *uncinatum* = *T. uncinatum*), collection date, Italian region of collection, spore morphology classification and the closest species match in the GenBank database. The accession numbers of the 27 sequenced samples are reported in the first column and an asterisk (*) highlights samples that present a mismatch in one of the three attributes (ascocarp, spore morphology, consensus taxa).

Sample ID/Query Accession Number	Recognized Species (Ascocarp)	Collection Date (Year)	Collection Site (Italian Region)	Spore Morphology Classification	Consensus Taxa/GenBank Accession Number
T1/MH992040	<i>T. aestivum</i>	2013	Molise	<i>T. aestivum</i>	<i>T. aestivum</i> /AY226026
T2/MH992046	<i>T. aestivum</i>	2013	Sardegna	<i>T. aestivum</i>	<i>T. aestivum</i> /AY226026
T3/MH992052	<i>T. aestivum</i>	2013	Sardegna	<i>T. aestivum</i>	<i>T. aestivum</i> /AY170350
T4/MH992059	<i>T. aestivum</i>	2013	Emilia Romagna	<i>T. aestivum</i>	<i>T. aestivum</i> /AY226022
T5 */MH992063	<i>T. uncinatum</i>	2013	Trentino Alto Adige	<i>T. mesentericum</i>	<i>T. mesentericum</i> /AF516775
T6/MH992064	<i>T. uncinatum</i>	2014	Lazio	<i>T. aestivum</i>	<i>T. aestivum</i> /AY226022
T7/MH992065	<i>T. mesentericum</i>	2013	Puglia	<i>T. mesentericum</i>	<i>T. mesentericum</i> /AF516778
T8/MH992066	<i>T. mesentericum</i>	2013	Lazio	<i>T. mesentericum</i>	<i>T. mesentericum</i> /AF516778
T9	<i>T. mesentericum</i>	2013	Lazio	<i>T. mesentericum</i>	
T10	<i>T. mesentericum</i>	2013	Lazio	<i>T. mesentericum</i>	
T11	<i>T. mesentericum</i>	2013	Lazio	<i>T. mesentericum</i>	
T12/MH992041	<i>T. mesentericum</i>	2013	Abruzzo	Uncertain	<i>T. mesentericum</i> /AF516778
T13	<i>T. mesentericum</i>	2013	Abruzzo	<i>T. mesentericum</i>	
T14/MH992042	<i>T. mesentericum</i>	2013	Abruzzo	<i>T. mesentericum</i>	<i>T. mesentericum</i> /AF516775
T15/MH992043	<i>T. mesentericum</i>	2013	Abruzzo	<i>T. mesentericum</i>	<i>T. mesentericum</i> /AF516769
T16/MH992044	<i>T. mesentericum</i>	2013	Lazio	<i>T. mesentericum</i>	<i>T. mesentericum</i> /AF516775
T17/MH992045	<i>T. mesentericum</i>	2013	Lazio	<i>T. mesentericum</i>	<i>T. mesentericum</i> /AF516775
T18	<i>T. mesentericum</i>	2013	Lazio	<i>T. mesentericum</i>	
T19	<i>T. mesentericum</i>	2013	Lazio	<i>T. mesentericum</i>	
T20	<i>T. mesentericum</i>	2013	Trentino Alto Adige	<i>T. mesentericum</i>	
T21	<i>T. mesentericum</i>	2013	Trentino Alto Adige	<i>T. mesentericum</i>	

Table 1. Cont.

Sample ID/Query Accession Number	Recognized Species (Ascomycota)	Collection Date (Year)	Collection Site (Italian Region)	Spore Morphology Classification	Consensus Taxa/GenBank Accession Number
T22/MH992047	<i>T. mesentericum</i>	2013	Trentino Alto Adige	<i>T. mesentericum</i>	<i>T. mesentericum</i> /AF516775
T23/MH992048	<i>T. mesentericum</i>	2013	Trentino Alto Adige	Uncertain	<i>T. mesentericum</i> /AF516775
T24	<i>T. mesentericum</i>	2013	Trentino Alto Adige	<i>T. mesentericum</i>	
T25	<i>T. mesentericum</i>	2013	Basilicata	<i>T. mesentericum</i>	
T26/MH992049	<i>T. mesentericum</i>	2013	Basilicata	<i>T. mesentericum</i>	<i>T. mesentericum</i> /AF516769
T27/MH992050	<i>T. mesentericum</i>	2013	Campania	<i>T. mesentericum</i>	<i>T. mesentericum</i> /AF516769
T28/MH992051	<i>T. mesentericum</i>	2013	Lombardia	<i>T. mesentericum</i>	<i>T. mesentericum</i> /AF516775
T29 *	<i>T. mesentericum</i>	2013	Emilia Romagna	<i>T. aestivum</i>	
T30 *	<i>T. mesentericum</i>	2013	Emilia Romagna	<i>T. aestivum</i>	
T31 *	<i>T. mesentericum</i>	2013	Emilia Romagna	<i>T. aestivum</i>	
T32*/MH992053	<i>T. mesentericum</i>	2013	Emilia Romagna	<i>T. aestivum</i>	<i>T. mesentericum</i> /AF516775
T33 *	<i>T. mesentericum</i>	2013	Emilia Romagna	<i>T. aestivum</i>	
T34	<i>T. mesentericum</i>	2014	Abruzzo	<i>T. mesentericum</i>	
T35/MH992054	<i>T. mesentericum</i>	2014	Abruzzo	Uncertain	<i>T. mesentericum</i> /AF516775
T36/MH992055	<i>T. mesentericum</i>	2014	Abruzzo	<i>T. mesentericum</i>	<i>T. mesentericum</i> /AF516769
T37/MH992056	<i>T. mesentericum</i>	2014	Lombardia	<i>T. mesentericum</i>	<i>T. mesentericum</i> /AF516775
T38/MH992057	<i>T. mesentericum</i>	2014	Lombardia	<i>T. mesentericum</i>	<i>T. mesentericum</i> /AF516769
T39/MH992058	<i>T. mesentericum</i>	2014	Lombardia	<i>T. mesentericum</i>	<i>T. mesentericum</i> /AF516775
T40 */MH992060	<i>T. mesentericum</i>	2014	Lombardia	<i>T. aestivum</i>	<i>T. aestivum</i> /AY226022
T41 *	<i>T. mesentericum</i>	2014	Lombardia	<i>T. aestivum</i>	
T42/MH992061	<i>T. uncinatum</i>	2013	Lazio	<i>T. aestivum</i>	<i>T. aestivum</i> /AY226026
T43/MH992062	<i>T. aestivum</i>	2013	Sardegna	<i>T. aestivum</i>	<i>T. aestivum</i> /AY226026

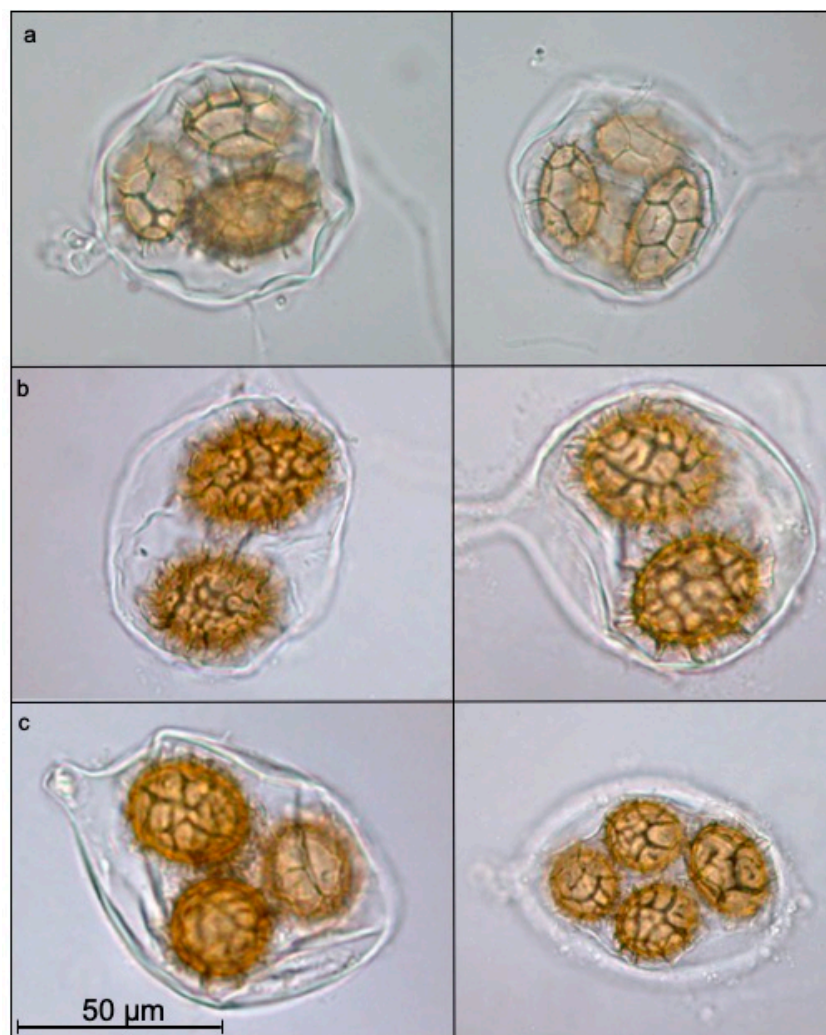


Figure 2. Ascospores recognized as belonging to *Tuber aestivum* (a) and *Tuber mesentericum* (b); (c) ascospores with uncertain spore morphology.

3. Results and Discussion

Our study started from the analysis of truffle specimens (samples T7, T8 and T12) collected in three different Italian regions. The aroma, the collection period, and ecological characteristics of the site where these truffles were collected were similar to those of *T. aestivum*. However their macroscopic and microscopic morphological features corresponded to those of *T. mesentericum*. Since these two truffle species usually live in the same soil environment, characterized by calcareous soils with sub-alkaline or neutral pH, their ascocarps are frequently mistaken for one another by collectors [2,36]. To better investigate the morphological and genetic variability of this truffle species, we gathered 43 samples of *T. mesentericum* and *T. aestivum* var. *uncinatum* from 10 more regions across Italy. The collected samples were classified by the truffle hunters as *T. aestivum* or *T. mesentericum* on the basis of main ascocarp characteristics, such as phenolic aromatic component, gleba color, presence of a basal depression or cavity, and the environmental characteristics of the collection site. The 43 truffles were subsequently classified according to their spore morphology [17,37]: 14 specimens were classified as *T. aestivum* and 26 as *T. mesentericum*, whereas three truffles were labeled as “uncertain” because their spore meshes were not well formed and they were not referable for certain to one of the two species (Table 1 and Figure 2). Sometimes, truffles that are not fully mature can have an incomplete episporium [38] that, in this case, can not be attributed to either *T. mesentericum* or *T. aestivum* spore types. According to our morphological analyses of the spores, truffle hunters misidentified eight truffles (Table 1). In particular, seven *T. mesentericum* ascocarps had

been classified as *T. aestivum* while only one *T. aestivum* var. *uncinatum* ascocarp had been recognized as *T. mesentericum*.

All the DNA sequences from the 27 truffles showed a BLAST match $\geq 99\%$ to *T. aestivum* or *T. mesentericum*. DNA sequencing confirmed the results provided by morphological analysis in 26 truffles, including those labeled as “uncertain” that had been correctly classified as *T. mesentericum* by the truffle hunters (Table 1). According to the DNA analysis, only one sample was wrongly identified by the spore morphology analysis (T32). In two other cases (T5 and T40), the DNA analysis agreed with the results of spore morphology analysis while confirming the incorrect identification made by truffle hunters. Our results confirmed how difficult it is to correctly identify *T. aestivum* and *T. mesentericum* truffles, even for experienced truffle hunters. It is worth highlighting that truffle hunters misidentified the samples because of the lack of the phenolic component in the aroma of the fresh ascocarps. It has been largely shown that truffle gleba represents a restricted habitat where bacteria, filamentous fungi, and yeasts can be harbored [39–45]. Although the role of microorganisms in truffle aroma was proven for *T. aestivum* [46], the same is not demonstrated for the phenolic component of *T. mesentericum*. Splivallo et al. [47] showed that certain bacteria promote the formation of thiophene derivatives, odorants unique to *T. borchii*. Recent findings showed that factors shaping the microbiome of *T. aestivum* ascocarps might differ based on local conditions, but unlike in other fungi, ascocarp maturation did not seem to influence the microbiome [48]. We hypothesize that, in addition to the truffle development stage, specific site conditions may also play a role in aroma definition, particularly for the presence of the phenolic component of *T. mesentericum*. Further study is necessary to define the relationships between the phenolic component in the truffle aroma and the ecology of *T. mesentericum* collection sites.

The EF1 α sequences produced in this study were used in two phylogenetic reconstructions. The first phylogenetic tree was constructed using only the sequences generated in this study (Figure 3), whereas in the second tree, all *T. aestivum* and *T. mesentericum* EF1 α sequences available in GenBank [26] were included (Figure 4).

Topology of the evolutionary tree in Figure 3 suggests the presence of two main clades: one grouping all *T. mesentericum* samples and the other clustering all *T. aestivum* samples; as expected, both clades are clearly separated from the *T. magnatum* outgroup. No sub-clades are evident in the *Tuber aestivum* clade, whereas three distinct sub-clades are marked in the *T. mesentericum* clade: sub-clade I and II belong to the same basal lineage, whereas sub-clade III is well separated from the other two. This evidence confirms what was proposed by Benucci et al. [14], who showed the presence of three distinct sub-clades in an ITS-based phylogeny of *T. mesentericum*. It is noteworthy that samples T7, T8, and T12 all belong to the *T. mesentericum* sub-clade III. Within-groups pairwise genetic distances were also calculated and compared considering both clade and sub-clade separation of samples. Results of the analysis (Table 2) showed that *T. aestivum* and *T. mesentericum* “sub-clade III” is the least diverse clade and sub-clade, respectively. In general, fairly low diversity was observed in all clades and sub-clades, while no diversity was observed in the *T. magnatum* one (data not shown).

The mantel test showed no significant correlation between the genetic distances between the truffles and the ecological data (Mantel $r = -0.025$, $p = 0.585$, permutations = 9999). The entanglement between the truffle phylogeny and the ecological dissimilarities was high (0.396) and the tanglegram showed almost no correspondence between the two trees besides sample T7 and T8 that fell in the same clade in both dendrograms (Figure 5).

Table 2. Estimates of evolutionary divergence over sequence pairs between groups. The number of base substitutions per site from averaging over all sequence pairs between groups are shown. Standard error estimate(s) are shown above the diagonal. Analyses were conducted using the Maximum Composite Likelihood model. The analysis involved 29 nucleotide sequences.

	<i>T. aestivum</i> Clade	Sub-Clade_III *	Sub-Clade_I *	Sub-Clade_II *	Outgroup
<i>T. aestivum</i> clade	-	0.0077	0.0077	0.0088	0.0118
sub-clade_III *	0.0334	-	0.0055	0.0066	0.0117
sub-clade_I *	0.0338	0.0205	-	0.0063	0.0121
sub-clade_II *	0.0409	0.0274	0.0225	-	0.0130
Outgroup	0.0651	0.0625	0.0664	0.0708	-

* Part of the *Tuber mesentericum* clade.

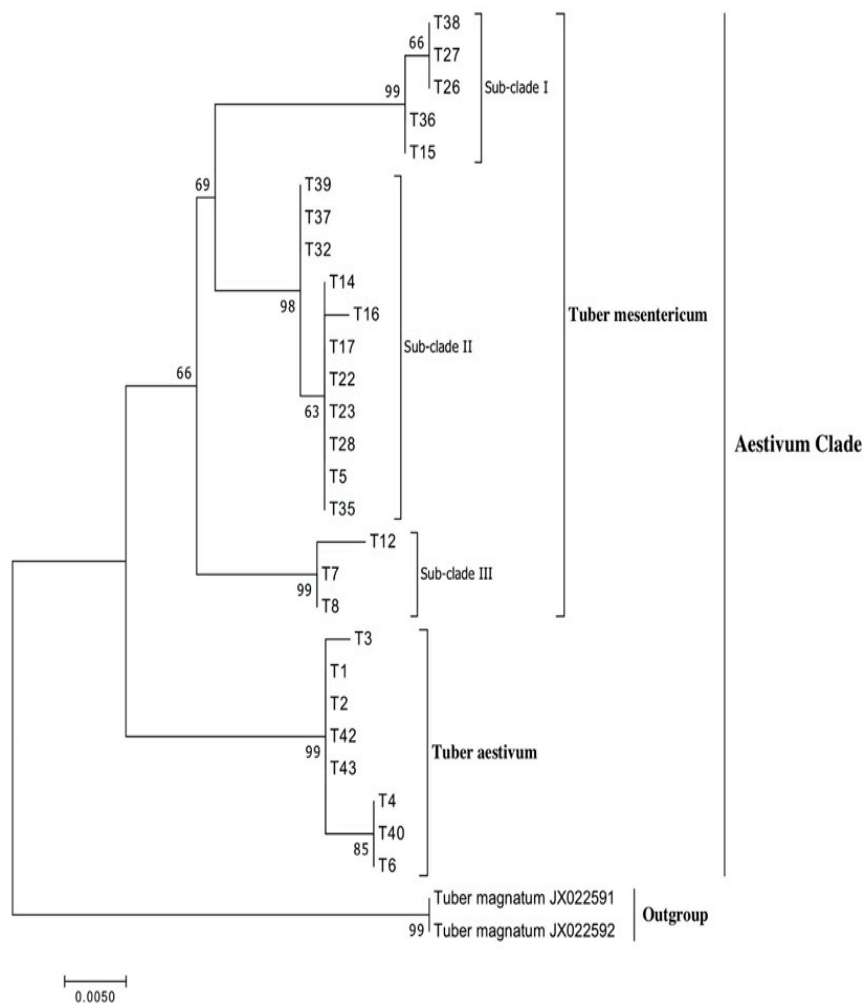


Figure 3. *Tuber mesentericum/aestivum* maximum likelihood phylogenetic tree realized with the EF1 α locus based on the Kimura two-parameter model [29]. Bootstrap values >60% are shown next to branching nodes. The tree is drawn to scale, with branch lengths measured in number of substitutions per site. The analysis involved 29 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 512 positions in the final dataset.

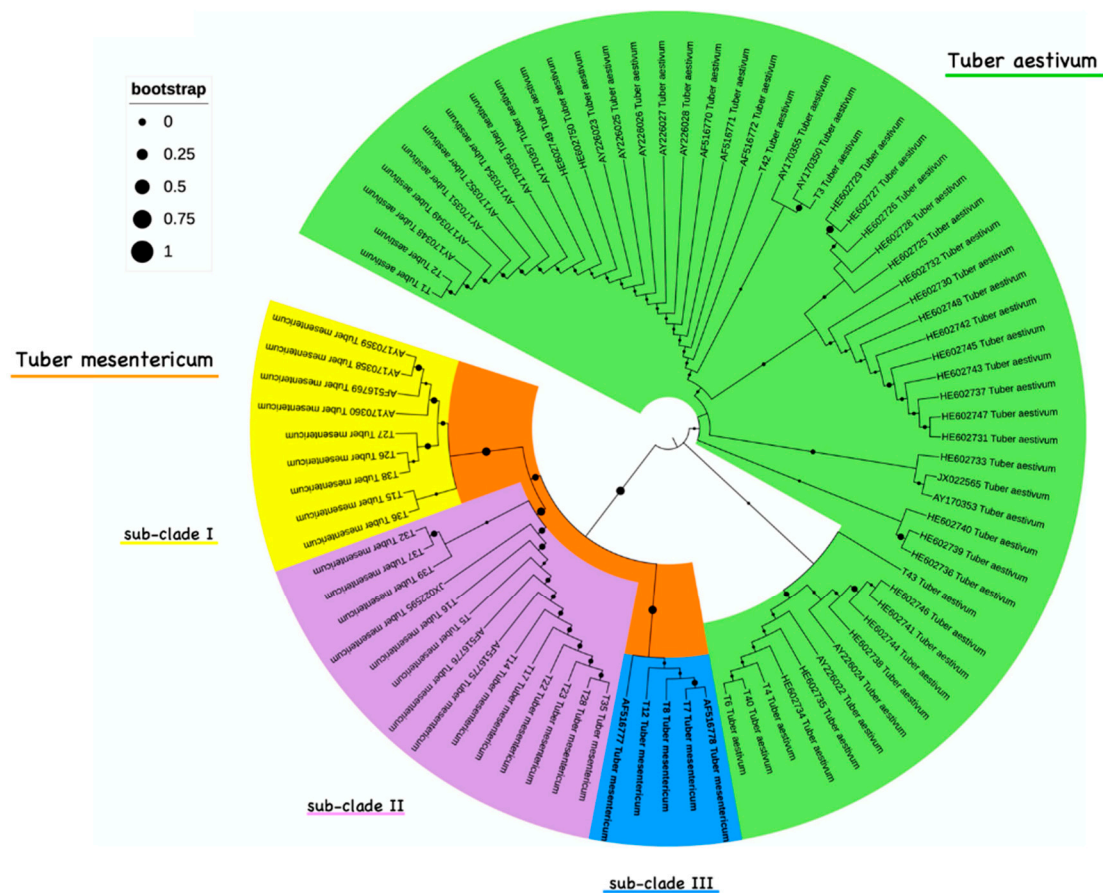


Figure 4. *Tuber mesentericum/aestivum* maximum likelihood phylogenetic tree based on the Kimura two-parameter model [29]; bootstrap values are shown as symbols on the branches. A discrete Gamma distribution [+G] was used to model evolutionary rate differences among sites. The analysis involved 83 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 465 positions in the final dataset. Sequence accessions and species are reported in the phylogenetic tree.

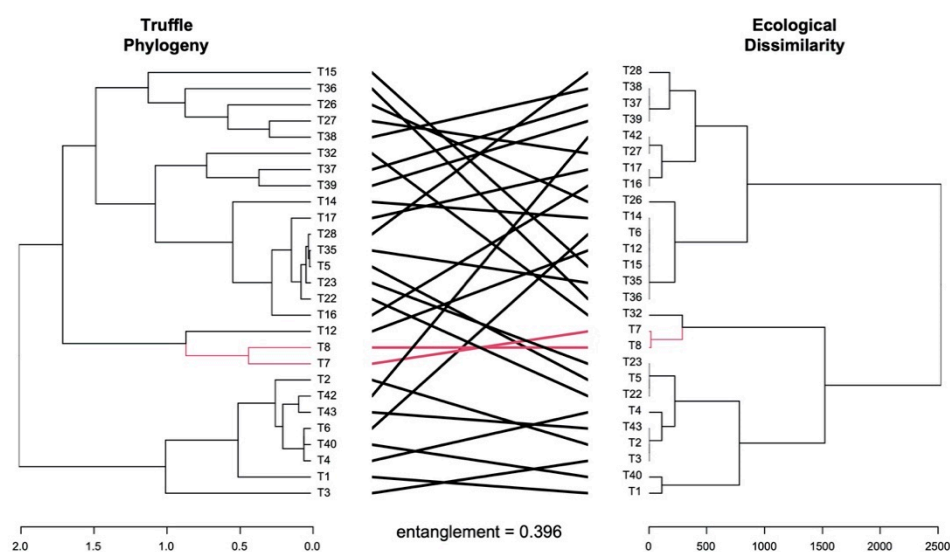


Figure 5. Tanglegrams showing correspondence between ecological differences at sampling sites (i.e., dissimilarities) and truffle phylogeny.

In order to better resolve the truffle phylogeny, we built a second maximum likelihood tree including all *T. mesentericum* and *T. aestivum* sequences available in GenBank [26]. Whereas a large number of EF1 α sequences of *T. aestivum* are present in GenBank, only 11 sequences of *T. mesentericum* exist. Both trees revealed the same topology, with two major clades that clearly separate *T. aestivum* from *T. mesentericum*. The latter included three main sub-clades. Sequences produced in this study cluster together with those with the highest similarity found in the GenBank database. In particular, sequences named AF516778 and AF516777 cluster together with samples T7, T8 and T12. Unfortunately, it was impossible to find out more information on the ascocarps that those two sequences belong to. Overall, information about the sampling location is lacking for most of the *T. mesentericum* downloaded sequences. This information was available only for sequence JX022595, which was isolated from a truffle collected in Sweden as reported by Bonito et al. [25] (Table S1). More information is needed regarding the aroma and morphological traits of the two truffles that grouped in sub-clade III in order to explore the potential reasons for the low genetic distance within the *T. aestivum* clade. In both *T. aestivum* and *T. mesentericum* clades, sequences obtained from samples collected in the same site cluster in different subclades, showing no geographic structure of the molecular data based on the selected locus (Figure 4). For example, *T. mesentericum* sub-clades I and II include sequences obtained from samples collected in Abruzzo, Lombardia, Basilicata, Campania, Emilia-Romagna, Lazio and Trentino Alto Adige (AY170358–AY170360, AF516769, AF516775–AF516776, JX022595).

Since the first ITS primers were published by White et al. [49], the ITS region of nrDNA has become the reference barcode region for the identification of fungi and phylogenetic studies [50–52]. A variety of species-specific primers were built for this region for the identification of *Tuber* spp. [21,53,54]. Nevertheless, ITS is difficult to align, which restricts its utility for phylogenetic reconstruction [55]. Therefore, new molecular markers were introduced for truffle multigene phylogeny studies [25,56], including the genetic locus elongation factor 1- α (EF1 α). To the best of our knowledge, this is the first study on *Tuber* spp. where the EF1 α locus is used to build a phylogeny of *T. mesentericum* and *T. aestivum*.

This study also confirms the high morphological and genetic similarity among *T. aestivum* and *T. mesentericum*, recommending the use of molecular tools for the characterization of these species. Currently, the high variability of the ITS region in *T. mesentericum* makes it impossible to design primers that are able to characterize all the genotypes of this species.

Bonito and colleagues [25], showed that *T. mesentericum* and *T. aestivum* cluster into one clade, together with *Tuber panniferum* Tul. & C. Tul. and *T. magnatum* in the ITS phylogeny of the *Tuber* genus. We showed that *T. mesentericum* and *T. aestivum* also have a high genetic variability in the EF1 α locus, corroborating previous findings on the presence of a “*Tuber aestivum*–*mesentericum* species complex”.

Moreover, we investigated the possible correspondence between the phylogenetic diversity of truffles and the ecological differences present at sampling sites using tanglegram analysis (Figure 5) and found no evident concordance. These results were corroborated by a non-significant Mantel test result.

The evidence shown in this work suggests that within the species complex, there are ecomorphs with intermediate morphological features located in a well-defined sub-clade. A wider collection of samples and a strong aroma characterization would help to more deeply investigate this species complex using, for example, a multiple gene phylogeny strategy. Moreover, studies on the *T. mesentericum* microbiome and aroma should be carried out to verify the influence of environmental conditions on the presence of the phenolic component. In future experiments, soil (e.g., soil texture, chemistry, temperature) as well as ecological (e.g., temperature, rain) traits of the sites where the truffles are collected should be included to possibly identify correlations between truffle genotypes and their ecology. *Tuber mesentericum* is not easily marketed because of its aroma and lack of mycorrhized seedlings with this truffle in the market. In the future, the design of species-specific primers on EF1 α or other loci could provide a tool for ascocarp characterization and truffle-inoculated seedling certification. This could also represent an opportunity to improve its cultivation. Finally, selecting *T. mesentericum*

ascocarps with an aroma more similar to *T. aestivum*, could open up new prospects for the market of this species.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1424-2818/12/9/0349/s1>, Table S1: GenBank accession information for downloaded EF1 α sequences, Table S2: Ecological information used in the tanglegram analysis.

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